Microsatellite variation in *Crotalus willardi obscurus* (I98011)

A report to the Arizona Game and Fish Department

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Isolation and Characterization of Microsatellite Loci from a Threatened Rattlesnake (New Mexico Ridgenose Rattlesnake, *Crotalus willardi obscurus*).

Microsatellite DNA loci have become the genetic marker of choice for studies of paternity, kinship, inbreeding, and fine scale population structure. Alleles are composed of tandem repeats of short sequences (typically 2–4 bp) of nucleotides. Microsatellites are often hypervariable in length (number of repeats) with each length polymorphism comprising a distinct allele for the locus (Tautz, 1989; Quellar et al., 1993). Microsatellites are selectively neutral, co-dominant, inherited in a simple Mendelian fashion, and assignable to specific loci (Quellar et al., 1993; Ashley and Dow, 1994). Furthermore, microsatellite loci often have utility in congeneric species and sometimes even more distantly related species. (e.g. Fitzsimmons et al., 1995; Gibbs et al., 1998; Bushar et al., 2001). Microsatellites have been characterized from a number of other snakes, including *Crotalus horridus* (Villareal et al., 1996), *Sistrurus catenatus* (Gibbs et al., 1998), *Hoplocephalus bungaroides* (Burns and Houlden, 1999), *Nerodia sipedon* (Prosser et al., 1999), *Thamnophis sirtalis* (McCracken et al., 1999), and *Natrix tessellata* (Gautscchi et al., 2000).

Here we report the isolation of 6 microsatellite loci from the New Mexico Ridgenose Rattlesnake *(Crotalus willardi obscurus*), and describe locus-specific characteristics within a single population. We also assess expected versus observed levels of heterozygosity, the utility of these loci for parentage analyses, and potential for cross-amplification in congeners. *Crotalus w. obscurus* is listed as "threatened" under the U.S. Endangered Species Act (U. S. Fish and Wildlife Service 1978). These markers will be used for analyses of variation within and among populations of this species, information essential to threat assessment and conservation planning.

METHODS AND MATERIALS

From 1993–1999 tissue was sampled from ca. 190 *C. w. obscurus* in the Animas Mountains, Hidalgo County, New Mexico. Of these, samples representing 54 individuals were randomly selected for genotyping in this preliminary survey of variability. In addition, we obtained tissue from a litter of eight offspring and their mother in the Peloncillo Mountains, Hidalgo County, New Mexico (Holycross, 2000). All snakes were marked for individual identification using passive integrated transponders (Jemison et al., 1995) to prevent unintentional resampling. Samples of ca. 0.1 ml whole blood were drawn from the caudal vein and immediately stored in either 1 ml 99% ethanol or 1 ml lysis buffer (Seutin et al., 1991). In the case of neonates, sloughed skins were used as a DNA source. Samples were stored at ambient temperatures (ca. 15–37 °C) for up to two months in the field and at <4 ˚C in the lab. We extracted DNA from ca. 200 µL of whole blood or 1–2 cm^2 of shed skin using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). DNA was resuspended in 1X TE (pH 7.5) and concentrations were estimated via agarose gel.

Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa* I, *Hae* III, *Bsr* B1, *Pvu* II, *Stu* I, *Sca* I, *Eco* RV). Fragments of 300 to 750 bp were adapted and subjected to magnetic bead capture (CPG, Inc., Lincoln Park, New Jersey), using biotinylated capture molecules. Libraries were prepared in parallel using Biotin-CA(15), Biotin-GA(15), Biotin-ATG(12) and Biotin-TAGA(8) as capture molecules in a protocol provided by the manufacturer. Captured molecules were amplified and restricted with *Hind*III to remove the adapters. Resulting fragments were ligated into the *Hind*III site of pUC19. Recombinant molecules were electroporated into *E. coli* DH5alpha. And 192 recombinant clones were randomly selected for sequencing. Sequences were obtained on an ABI 377, using ABI Big Dye

terminator cycle sequencing methodology. Primers flanking the repetitive elements were designed using Oligo 4.0 software (National BioSciences Inc., USA) and oligonucleotides were synthesized by MWG Biotech (USA). The forward primer for each pair was labeled with a fluorescent molecule.

PCR amplification for polymorphism assessment was performed in a 20 μL reaction volume containing 10 ng of genomic DNA, 20.0 mM Tris-HCl (pH 9.0), 8.5 mM NaCl, 10 mM KCl, 10.0 mM $(NH_4)^2SO_4$, 2.0 mM $MgSO_4$, 0.1% (w/v) Triton X-100, 0.5% (w/v) Ficoll, 10 picomole each of forward and reverse primer and 0.5 units of Taq DNA polymerase using a PTC-100™ Programmable Thermal Controller (MJ Research Inc.). Amplification was performed under the following conditions: 35 cycles at 95 °C for 30 s, the locus-specific annealing temperature (Table 1) for 30 s, and 72 °C for 30 s. Before the first cycle, a prolonged denaturation step (95 °C for 2 min) was included. Amplified products were diluted with double-distilled water containing GENESCAN-500XL (TAMRA) Size Standard (PE Biosystems) and genotyped on an ABI Prism 377 Genetic Analyser using GeneScanAnalysis® Software version 3.1 and Genotyper® version 2.5 software (PE Biosystems). Observed and expected heterozygosities and likelihood ratio tests for Hardy-Weinberg equilibrium at each locus were conducted using Popgene version 1.32 (Yeh and Boyle, 1997). Exclusionary power for parentage analyses was conducted using Cervus 2.0 (Marshall et al., 1998).

RESULTS AND DISCUSSION

Repetitive sequences were observed in 35 of 192 clones. Primers were designed within flanking sequence and annealing temperatures optimized for 15 clones: 14 of these amplified products. We tested for polymorphism using genomic DNA from 10 individuals. Six of these loci produced scoreable, polymorphic products and were used to survey variation in the Animas

Mountain population. Rejected loci (n = 8) were homozygous or difficult to score due to stutter peak configurations. Annealing temperatures, repeat motifs, and primer sequences for each locus are provided in Table 1. Of the 6 microsatellites chosen, four were dinucleotides (*Cw*A14, *Cw*A29, *Cw*B6, *Cw*B23) and two were trinucleotides (*Cw*C24, *Cw*D15). All loci consist of uninterrupted strings of one or two motifs (Table 1). Analysis of a litter of eight *C. w. obscurus* and their mother (from the Peloncillo Mountains) as well as litters of five *Crotalus atrox* and four *Crotalus scutulatus* and their mothers, are not inconsistent with patterns of Mendelian segregation. All offspring share at least one allele with their mother. A maximum of two additional alleles other than those observed in the mother were present among offspring at each locus, which is not inconsistent with single male paternity.

Surveys of 53 to 54 *C. w. obscurus* (sample size varies among loci) from the Animas population revealed 5–24 alleles per locus (Table 1). Although these loci did not exhibit strongly disjunct distributions, most exhibited small gaps between some adjacent alleles (Fig. 1). Specifically, the largest gaps between alleles were six (*Cw*A29) and five (*Cw*A14) unoccupied potential allelic states. All other loci were characterized by gaps < 4 repeat units between alleles. These distributional patterns are not inconsistent with stepwise (Valdes et al., 1993) or two-phase (Di Rienzo et al., 1994) models of mutation in microsatellites.

Across all loci, mean observed heterozygosity (0.696) approximates mean expected heterozygosity (0.714), and no significant deviations from Hardy–Weinberg expectations were detected (Table 1). These results suggest that the potential for null alleles is low. F_{IS}, a measure of the extent of nonrandom association of alleles within a population, is likewise low (Table 1). we assessed the utility of these loci for parentage analyses by calculating exclusionary power using this dataset of 54 individuals from the Animas population and the program Cervus 2.0

TABLE 1. Characteristics of six microsatellite loci developed from *Crotalus willardi obscurus* genomic DNA. F = forward primer and R = reverse primer. * = flourescently labeled primer. T_m = annealing temperature (ºC) used to assay variation. A = number of alleles detected. N = number of individuals genotyped. Size refers to the range of allele lengths (bp) detected. H_{exp} = expected heterozygosity (Levene, 1949) and H_{obs} = observed heterozygosity. P = significance level of likelihood ratio (G^2) tests for Hardy-Weinberg equilibrium. F_{IS} = Wright's (1978) fixation index.

Locus	Repeat Motif	Primer sequence (5' → 3')	T_m	Size	A	N	H_{exp}	H_{obs}	P	F_{IS}
*Cw*A14	$(AC)_{24}$	F: GGGGAGGTAGGGAGGTCAG*	62	147–	7	54	0.775	0.68	(0.86)	0.108
		R: AGGGGAAAAGATGCTGTGAG		175				5		
*Cw*A29	$(AC)_{13}$	F: TCCCCTTCCAACCCCCAGA*	60	160–	5	54	0.351	0.31	(0.94)	0.095
		R: CAGAGGAGACGAGACAGATAG		190				5		
*Cw*B6	$(GA)_{19}$	F: CTCTTTTACGCCCACCACTTTA*	56	122–	5	54	0.726	0.79	(0.95)	-
		R: CCCCGCTAACCTTTGCTCAG		130				6		0.107
*Cw*B23	$(TG)_{18}(AG)_{22}$	F: TGGTGTCATCTGGAGTTAAATC*	60	225–	12	53	0.857	0.75	(0.58)	0.111
		R: GCTTTTGTTTATATGGAGAGTCG		271				5		
*Cw*C24	$(CTT)_{49}$	F: ATTGGATAGAAGTAGTTTTGGTA*	62	235–	24	53	0.921	0.90	(1.00)	0.007
		R: CCCCCCTTTTTTTATGGCAGC		313				6		
*Cw*D15	$(CAT)(TAT)(CAT)_{14}$	F: TAATGTTGTAAGCCACCTAGAAT*	58	138–	5	53	0.654	0.71	(0.48)	-
		R: TTCTTCAAAGCACATAACACATC		159				7		0.107

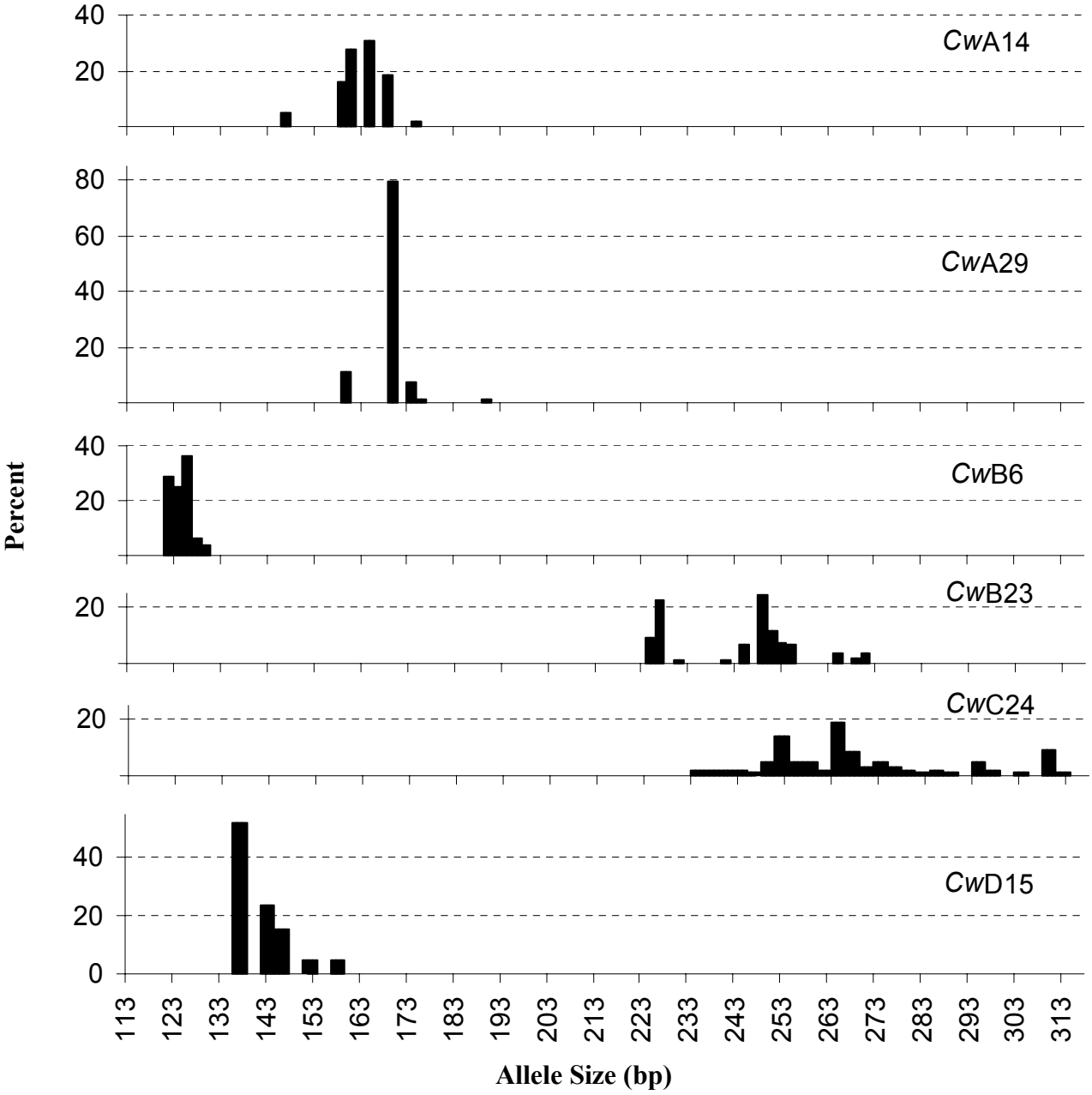

FIG. 1. Allele distribution at 6 microsatellite loci in the Animas population of *Crotalus willardi obscurus*.

(Marshall et al., 1998). Across all loci, exclusionary power for the first parent is 0.96, whereas if the genotype of one parent is known, exclusionary power for the second parent is 0.99.

We tested the utility of the primers for cross-amplification of homologous loci in four other rattlesnake species using annealing temperatures reported in Table 1. Of 24 locus/species combinations, only three failed to amplify and 18 of the remaining 21 locus/species combinations produced two or more size products (Table 2). Low levels of variability in *C. atrox* and *C. scutulatus* at some loci may be due to a high degree of relatedness among individuals sampled (Table 2). Additional surveys of unrelated individuals and testing using a variety of PCR conditions are necessary for more representative characterization of cross-amplification and levels of polymorphism in these locus/species combinations. Although loci amplified with heterospecific primers often exhibit reduced variability (Moore et al., 1991; Primmer et al., 1996), these data suggest this set of variable microsatellite loci may prove useful for a variety of population-level and relatedness analyses in *C. willardi* and other rattlesnakes.

TABLE 2. Results of cross-amplification experiments. Sizes of amplified products (in bp) are indicated for each locus. *N* = number of individuals genotyped. *Crotalus atrox* samples consisted of a mother and five offspring as well as one unrelated individual. *Crotalus scutulatus* consisted of a mother and four offspring. *Crotalus lutosus* and *Crotalus tigris* samples consist of unrelated individuals from a single population. Annealing temperatures are the same as those reported in Table 1.

		Locus					
Species	*n*	*Cw*A14	*Cw*A29	*Cw*B6	*Cw*B23	*Cw*C24	*Cw*D15
C. atrox	7	159, 169, 171, 175	170, 182, 184	98, 104	217, 227, 245, 251	–	125
C. lutosus	5	155, 159	166, 172	–	215, 217, 221, 227, 235	–	132, 135, 144, 147, 153
C. scutulatus	5	157, 161	162,164, 166	106	221, 227, 231, 233, 235, 241, 245	259, 262, 298, 307	129, 132, 156
C. tigris	6	157, 165, 167, 169	160, 162	127, 135	233, 241, 243	205, 253, 259, 262, 265, 277, 286, 289, 298	126

Genetic variation and population structure in a threatened rattlesnake (New Mexico Ridgenose Rattlesnake, *Crotalus willardi obscurus*)

Conservation of endangered species requires identification of independently evolving entities or "evolutionarily significant units" (ESUs; Waples 1995) in order to delineate populations and preserve evolutionary history and trajectories. In the case of small isolated populations, additional questions are of interest, such as structure within populations, past demographic history (e.g. bottlenecks) and migration rates. Molecular genetic markers, particularly microsatellite DNA loci, are especially well suited to analysis of fine-scale population structure and differentiation. Microsatellite DNA is often hypervariable in length (number of repeats) with each length polymorphism comprising a distinct allele for the locus (Tautz, 1989; Quellar et al., 1993). Microsatellites are neutral, co-dominant, inherited in a simple Mendelian fashion, and assignable to specific loci (Quellar et al., 1993; Ashley and Dow, 1994). This suite of characteristics renders them especially useful for studies of relatedness, inbreeding, and recovering recent evolutionary history and extant genetic patterns among closely related populations. They are thus suitable for defining ESUs. Inherent to the concept of ESUs is the ideal of perpetuating adaptive variation among populations. Although microsatellites are neutral, they evolve at rates that approximate rates of evolution of quantitative genetic variation (with potentially meaningful adaptive relevance) and thus can provide insight to the potential for local adaptation (Hedrick et al., 2001).

Crotalus w. obscurus (New Mexico Ridgenose Rattlesnake), is restricted to Madrean montane woodland communities in the Sierra San Luis (Mexico) and in the neighboring Animas and Peloncillo Mountains (United States). Low elevation passes separate the Sierra San Luis

from the Animas and Peloncillo Mountains (Fig. 2), but may have provided corridors for past genetic exchange between the Sierra San Luis and the two northern mountain ranges. The broad Animas Valley separates the Peloncillo and Animas Mountains and is probably a complete barrier to extant migration. Different histories of isolation and rates of reduction in population size may have contributed to different genetic circumstances for each population. Variation was found at three allozyme loci (of 33 screened) in the San Luis population ($N = 6$). However, these loci were monomorphic in small samples from the Animas ($N = 1$) and Peloncillo ($N = 3$; mother and two offspring) populations (Barker, 1992). Several authors have suggested various phylogeographic and biogeographic scenarios to explain the current distribution of *C. willardi* (Fowlie, 1965; Klauber, 1972; McCranie and Wilson, 1987; Barker, 1992). All are variations on a theme of northward expansion of range followed by subsequent vicariance. A fossil from the San Pedro river valley tentatively identified as *Crotalus willardi* (Mead, 1975) lends credence to the hypothesis that *C. w. obscurus* occupied wooded Pleistocene valleys (Barker, 1992) prior to its current insular distribution. *Crotalus w. obscurus* was listed is 'threatened' under the Endangered Species Act (United States Fish and Wildlife Service, 1978). A Species Recovery Plan (Baltosser and Hubbard, 1985) recommended *in situ* study and establishment of a captive breeding program based on extremely limited information.

Delineation of patterns of genetic variation within and among populations will allow management agencies to make informed decisions regarding jeopardy rulings, allocation of conservation resources, captive breeding programs, formulation of recovery plans, and translocation of animals. Description of geographic patterns of genetic variation may also help

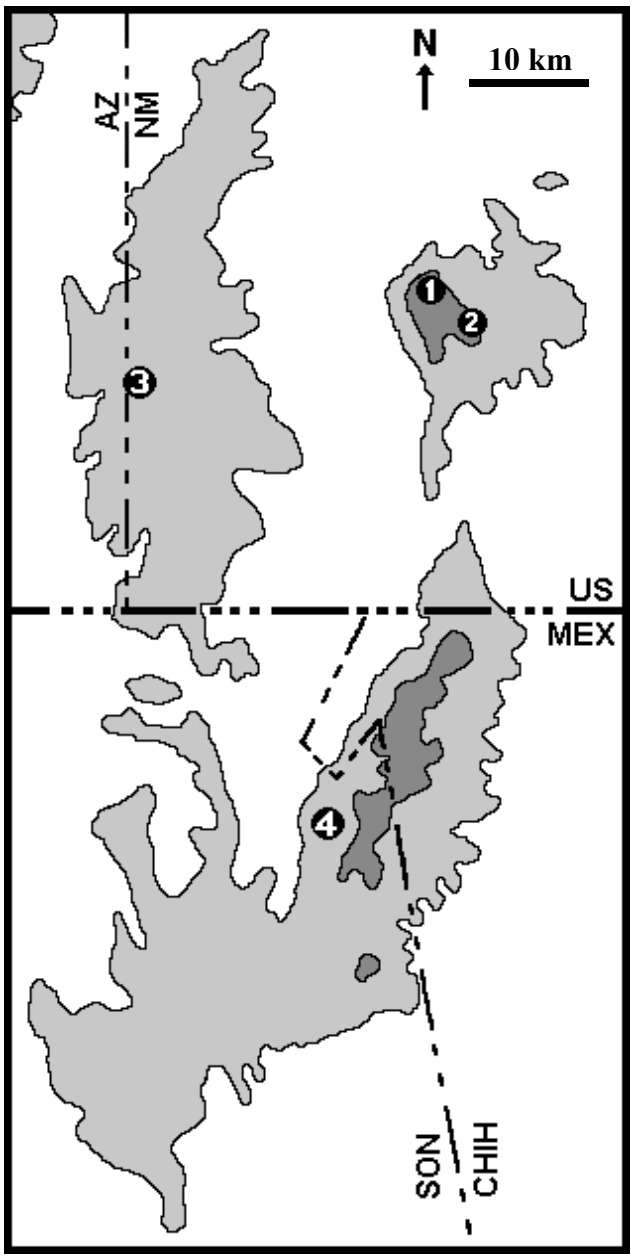

FIG. 2. Location of *Crotalus willardi obscurus* sampling localities in West Fork Canyon, Animas Mountains (1) and Indian Creek Canyon, Animas Mountains (2), Peloncillo Mountains (3), and Sierra San Luis (4). Light and dark gray isopleths delineate Madrean Evergreen Woodland and Petran Montane Conifer Forest, respectively (modified from Brown and Lowe, 1994).

identify source populations of expatriated animals, thus aiding in law enforcement activities and/or repatriation efforts. Currently, Animas Mountain is the only "critical habitat" listed for *C. w. obscurus*. Critical habitat has not been designated for the Peloncillo population, the smallest of the three populations, and the only population occurring on public land. Evidence of independent evolution among populations is necessary before each population can be regarded as a discrete population segment (DPS) under the Endangered Species Act. Designation of each population as a DPS is required in order to designate separate critical habitat for each population. This work is part of a long-term study of the conservation biology of the three known populations of this threatened species (Holycross and Goldberg, 2001; Smith et al., 2001; Holycross et al., In press).

MATERIALS AND METHODS

Populations Sampled

We sampled tissue from *C. w. obscurus* in the Animas Mountains (B = 54), Peloncillo Mountains (N = 18), and Sierra San Luis (N = 29) from 1993–1999 (Fig. 2). In the Animas Mountains, we sampled intensively from two sites in separate canyons. Tissues in the Peloncillo Mountains were collected from three separate drainage systems. In the Sierra San Luis tissues were collected from a single locality in a tributary to Cajon Bonita. Herein, “samples” refers to each of the four sampling localities, while “populations” refers to all samples from each mountain range (i.e. Animas samples combined). To prevent unintentional resampling we marked all snakes for individual identification using passive integrated transponders (Jemison et al., 1995). We sampled ca. 0.1 ml whole blood from the caudal vein and immediately stored this in either 1 ml 99% ethanol or 1 ml lysis buffer (Seutin et al., 1991). In a few cases, skins

sloughed during holding were retained as a source of DNA. Due to logistic constraints, samples were stored at ambient temperatures (ca. 15–37 °C) for up to two months in the field. In the laboratory, samples were stored at <4˚C, for up to several years.

Laboratory Methods

We extracted DNA from ca. 200 µL of whole blood or 1–2 cm^2 of shed skin using a standard phenol-chloroform extraction protocol (Sambrook et al., 1989). DNA was resuspended in 1X TE (pH 7.5) and concentrations were estimated via agarose gel. Individuals were then genotyped at each of nine microsatellite loci; six (*Cw*A14, *Cw*A29, *Cw*B6, *Cw*B23, *Cw*C24, *Cw*D15) developed from *Crotalus willardi obscurus* genomic DNA and three loci (Scµ01, Scµ07, Scµ11) from *Sistrurus catenatus* (Gibbs et al., 1998). Two loci are trinucleotide repeats (*Cw*C24, *Cw*D15) and the remaining seven are dinucleotide repeas. PCR amplification for polymorphism assessment was performed in a 20 µL reaction volume containing 10 ng of genomic DNA, 20.0 mM Tris-HCl (pH 9.0), 8.5 mM NaCl, 10 mM KCl, 10.0 mM $(NH_4)^2SO_4$, 2.0 mM $MgSO_4$, 0.1% (w/v) Triton X-100, 0.5% (w/v) Ficoll, 10 picomole each of forward and reverse primer and 0.5 units of Taq DNA polymerase thermotreatment on a PTC-100™ Programmable Thermal Controller (MJ Research Inc.): 35 cycles at 95 °C for 30 s, the locus-specific annealing temperature for 30 s, and 72 °C for 30 s. A prolonged denaturation step (95 °C for 2 min) was included before the first cycle. Amplified products were diluted with double-distilled water containing GENESCAN-500XL (TAMRA) Size Standard (PE Biosystems) and analyzed on an ABI Prism 377 Genetic Analyser using GeneScanAnalysis® Software version 3.1 and Genotyper® version 2.5 software (PE Biosystems).

Analysis of Variation

Initial manipulations of data, data checking, and creation of input files was conducted using the MS Toolkit macro for Excel (Park, 2001). We calculated allele frequencies and expected and observed heterozygosity using POPGEN version 1.32 (Yeh and Boyle, 1997). Randomization tests for Hardy-Weinberg equilibrium were conducted using GENEPOP version 3.1 (Raymond and Rousett, 1995). We tested each locus in each of the three populations (27 comparisons) and in each of the samples from the Animas Mountains (18 comparisons). GENEPOP combines these probabilities to test populations across loci or one locus across populations. Critical values for statistical significance were Dunn-Šidák adjusted (Sokal and Rohlf, 1995) for multiple comparisons.

We quantified differentiation between populations using several methods. We tested for heterogeneity in allele frequencies among and between populations (and between Animas samples) using the exact test in GENEPOP. Proportions of private alleles in each population were calculated by hand. Fixation (F_{ST}; Weir and Cockerham, 1984) and distance indices (D; Nei, 1978) based on the infinite alleles model (IAM) were calculated using GENEPOP and POPGEN, respectively. PHYLIP (Felsenstein, 1993) was used to construct a UPGMA (unweighted pair-group method using arithmetic averages) tree representing the phylogenetic relationships among sampling sites based on genetic distance. FSTAT version 2.9.1 (Goudet, 1995) was used to calculate F-statistics (F_{IS}, F_{ST}, and G'_{ST}) and test F_{ST} estimators for significance. Nm was estimated using the private alleles method (Barton and Slatkin, 1986) in GENEPOP.

We used the program BOTTLENECK version 1.2.02 (Cornuet and Luikart, 1996) to assess statistical evidence for a past bottleneck in each of the three populations. Due to small

sample size and few loci, we used the Wilcoxon signed-ranks option in BOTTLENECK to test for differential rate of decline in number of alleles relative to heterozygosity under the two-phase mutation model (TPM) in a population at equilibrium (H_{eq}). We also assayed for evidence of recent bottlenecks using *M* (Garza and Williamson, 2001), a ratio of the number of alleles detected relative to range in allele sizes. We calculated *M* for each population using the six loci (*Cw*A14, *Cw*A29, *Cw*B6, *Cw*B23, *Cw*C24, *Cw*D15) that conformed to calculation criteria and were developed from *C. w. obscurus* genomic DNA.

RESULTS

Allele frequencies for all locus-sample combinations are reported in the Appendix. Genetic characteristics and summary statistics for each locus-population combination are provided in Table 3. Twenty-five (93%) of 27 locus/population combinations were polymorphic. The only monomorphic exceptions were the San Luis and Peloncillo populations at *Scu*07. *Scu*07 had the lowest expected and observed heterozygosity, the lowest average number of alleles across populations (1.3), and just two alleles detected overall (Table 3). *Cw*C24 had the highest expected and observed heterozygosity, the highest average number of alleles across populations (19.0), and the most alleles (across populations) with 30 alleles occupying 31 possible trinucleotide positions between 229–319 bp (Table 3).

Populations

Although all populations contained substantial genetic variation, the Peloncillo population was less variable than the Animas or San Luis populations. The Peloncillo population

TABLE 3. Genetic characteristics of three populations (and two subsamples) of *Crotalus willardi obscurus* at nine microsatellite loci. ICC and WFC indicate Indian Creek Canyon and West Fork Canyon demes. *N* = number of individuals genotyped. *A* = number of alleles detected. 'Size' = the range of allele lengths (bp) detected. H_{obs} = observed heterozygosity and H_{exp} = expected heterozygosity (Levene, 1949). *P* = significance level of randomization tests for Hardy-Weinberg equilibrium. F_{IS} = Wright's (1978) fixation index.

	Population				
Locus	San Luis	Peloncillo	Animas (pooled)	Animas (WFC)	Animas (ICC)
***Cw*A14**					
N	29	18	54	30	24
A	8	3	7	6	6
Size	147–167	165–169	147–175	147–175	147–169
H_{obs}	0.793	0.444	0.685	0.633	0.750
H_{exp}	0.848	0.513	0.775	0.758	0.778
P	0.138	0.376	0.658	0.394	0.774
F_{IS}	0.065	0.137	0.117	0.167	0.036

continued

TABLE 3, continued

***Cw*A29**					
N	29	18	54	30	24
A	11	5	5	3	5
Size	160–196	172–190	160–190	160–174	160–190
H_{obs}	0.759	0.833	0.315	0.300	0.333
H_{exp}	0.752	0.675	0.351	0.297	0.420
P	0.548	0.881	0.473	0.593	0.339
F_{IS}	–0.009	-0.244	0.104	-0.012	0.210
***Cw*B6**					
N	29	18	54	30	24
A	7	4	5	5	4
Size	98–128	98–134	122–130	122–130	122–128
H_{obs}	0.759	0.444	0.796	0.767	0.833
H_{exp}	0.756	0.592	0.726	0.740	0.705
P	0.728	0.445	0.964	0.997	0.758
F_{IS}	-0.003	0.255	-0.098	-0.037	-0.187

continued

TABLE 3, continued

***Cw*B23**					
N	29	18	53	29	24
A	13	7	12	10	11
Size	245–275	233–271	225–271	225–271	225–271
H_{obs}	0.724	0.667	0.755	0.793	0.708
H_{exp}	0.868	0.783	0.857	0.849	0.865
P	0.025	0.032	0.070	0.459	0.006
F_{IS}	0.168	0.152	0.121	0.067	0.185
***Cw*C24**					
N	29	18	53	30	23
A	18	15	24	18	22
Size	229–319	229–304	235–313	235–313	235–310
H_{obs}	0.966	0.778	0.906	0.900	0.913
H_{exp}	0.905	0.932	0.921	0.902	0.942
P	0.718	0.207	.423	0.286	0.300
F_{IS}	-0.068	0.169	0.017	0.003	0.031

continued

TABLE 3, continued

***Cw*D15**					
N	29	18	53	29	24
A	7	5	5	5	5
Size	132–156	138–156	138–159	138–159	138–159
H_{obs}	1.000	0.889	0.717	0.793	0.625
H_{exp}	0.820	0.735	0.654	0.684	0.614
P	0.275	0.981	0.814	0.492	0.842
F_{IS}	-0.225	-0.217	-0.097	-0.162	-0.018
Scμ01					
N	28	18	53	29	24
A	9	4	11	9	10
Size	158–208	178–204	166–204	166–204	166–204
H_{obs}	0.786	0.667	0.906	0.966	0.833
H_{exp}	0.781	0.560	0.849	0.828	0.864
P	0.054	0.616	0.270	0.583	0.312
F_{IS}	-0.006	-0.196	-0.068	-0.169	0.037

continued

TABLE 3, continued

Scμ07					
N	28	13	52	29	23
A	1	1	2	2	2
Size	146	146	146–148	146–148	146–148
H_{obs}	0	0	0.539	0.621	0.435
H_{exp}	0	0	0.505	0.508	0.510
P	—	—	0.781	0.278	0.676
F_{IS}	—	—	-0.067	-0.226	0.151
Scμ11					
N	27	18	53	29	24
A	12	6	10	9	10
Size	170–216	194–210	172–214	172–212	172–214
H_{obs}	0.852	0.667	0.736	0.759	0.708
H_{exp}	0.851	0.735	0.861	0.836	0.892
P	0.210	0.873	0.022	0.024	0.135
F_{IS}	-0.002	0.095	0.146	0.093	0.209

continued

TABLE 3, continued

All loci					
N (mean)	28.6	17.4	53.2	29.4	23.8
A (mean)	9.6	5.6	9	7.4	8.3
H_{obs}	0.738	0.599	0.706	0.726	0.682
H_{exp}	0.731	0.614	0.722	0.711	0.732
P	0.063	0.516	0.300	0.360	0.176
F_{IS}	-0.009	0.025	0.022	-0.021	0.070

averaged 5.6 alleles/locus, compared with 9.0 and 9.6 alleles/locus in the Animas and San Luis populations. Average expected heterozygosity ranged from a low of 0.61 in the Peloncillo population to 0.72 and 0.73 in the Animas and San Luis populations, respectively. After correction of critical values for multiple comparisons, no significant departures from Hardy–Weinberg expectations were detected in locus specific tests within populations (Table 3), in populations (across loci), or at individual loci (across populations). We also calculated the "inbreeding coefficient" (F_{IS}), which is a measure of the heterozygote deficit within populations, for each locus in each population and sample (Table 3). Overall F_{IS} did not significantly differ from zero (bootstrapping across loci 99% confidence intervals: –0.075 to 0.089). We tested for bottlenecks in each population using the Wilcoxson signed-rank test to compare observed heterozygosity to H_{eq} under the TPM (Cornuet and Luikart, 1996) and detected significant differences only in the Peloncillo population ($P < 0.02$). Evidence of bottlenecks was also assessed by calculating M (Garza and Williamson, 2001) for each population: $M = 0.653 \pm 0.059$ in the San Luis population, 0.632 ± 0.108 in the Animas population, and 0.559 ± 0.114 in the Peloncillo population.

The Animas Mountains population was sampled from two geographically discrete canyons in order to facilitate analysis of genetic patterns within mountain ranges. Allele frequencies in the two Animas samples did not significantly differ in any locus-specific comparison after correction for multiple comparisons (Table 4). Nor did they significantly differ when combined across loci ($P = 0.051$). While there were unique alleles in each of these samples (when contrasted exclusively with each other), the frequency of unique alleles was generally low and these typically appeared at loci where the number of alleles detected approached the sample size (Appendix). Observed heterozygosity was slightly lower than, but not significantly different

from expected heterozygosity in the population overall (Table 3), and F_{IS} did not significantly differ from zero. Genetic distance between the WFC and ICC samples in the Animas Mountains was low (0.035) as was F_{ST} (0.004). Measures of migration based on pairwise F_{ST} (that require a number of biologically unreasonable assumptions; Whitlock and McCauley, 1999) estimate *Nm* at 62.3. Using the private allele method to estimate migration rates between the two samples we found *Nm* = 3.04, after correction for size (Barton and Slatkin, 1986).

Population Differentiation

Allele frequencies among the three populations significantly differed at all loci ($P = 0$ for all comparisons) and when combined across loci ($P = 0$; Table 4). In pair-wise comparisons of allele frequencies at each locus, most tests (25 of 27) were highly significant ($P = 0$). Two tests were non-significant after critical values were adjusted for multiple comparisons: the Peloncillo and San Luis populations at *Cw*B6 and the Animas and Peloncillo populations at *Cw*C24 (Table 4). Unique and private alleles were detected in all three populations (Table 5). Private alleles are unique alleles with frequencies ≥ 5%. In the Animas and San Luis populations, 30% and 37 % of alleles were unique and 12% and 20% of alleles were private. In contrast, 14% of alleles in the Peloncillo population were unique and 8% of alleles were private. The relatively low proportion of unique alleles in the Peloncillo population may be partly accounted for by low sample size, such that alleles occurring at low frequency (<5 %) were not detected. However, in both the San Luis and Animas populations, 5% of all alleles were private alleles that occurred at frequencies of ca. 20% or higher, whereas none of the private alleles in the Peloncillo approached these proportions. Furthermore, in a comparison of 18 individuals drawn at random from each population, five private alleles were detected in the Peloncillo population, whereas 10

TABLE 4. Significance levels of tests of Ho: allele frequencies do not differ among/between populations. A = Animas, P = Peloncillo, and S = San Luis populations. ICC and WFC indicate the Indian Creek Canyon and West Fork Canyon samples in the Animas Mountains.

	A v. P v. S	A v. P	P v. S	S v. A	ICC v. WFC
Locus	*P*	*P*	*P*	*P*	*P*
*Cw*A14	0.000	0.000	0.000	0.000	0.089
*Cw*A29	0.000	0.000	0.000	0.000	0.422
*Cw*B6	0.000	0.000	0.025	0.000	0.192
*Cw*B23	0.000	0.000	0.000	0.000	0.293
*Cw*C24	0.000	0.030	0.000	0.000	0.228
*Cw*D15	0.000	0.000	0.000	0.000	0.256
Scμ01	0.000	0.000	0.000	0.000	0.021
Scμ07	0.000	0.000	—	0.000	0.843
Scμ11	0.000	0.000	0.000	0.000	0.246
Combined	**0.000**				**0.051**

and 18 private alleles were detected in the Animas and San Luis populations.

Genetic distance between mountain populations (Table 6) ranged from 0.446 (Peloncillo - San Luis) to 0.780 (Peloncillo- Animas). Pair-wise distances between all four samples (Table 6) were used to construct a UPGMA phylogenetic tree to summarize relationships among samples (Fig. 3). F_{ST} (Weir and Cockerham, 1984) was 0.1594 and G'_{ST} (Nei, 1987) was 0.164. Bootstrapping across loci, the 99% confidence interval for overall F_{ST} ranged from 0.086–0.255. Pairwise F_{ST} estimates for each locus are presented in Table 6. Using the private allele method (Barton and Slatkin, 1986) and after correction for size, $Nm = 0.66$ among the three populations.

DISCUSSION

Populations

On the whole, these loci exhibit high levels of variability, rendering them useful for investigations of population structure and differentiation. All populations conformed to Hardy-Weinberg equilibrium, facilitating uncomplicated analysis and interpretation. For example, conformation to Hardy-Weinberg expectations and absence of significant F_{IS} values, suggest that null alleles, a common problem in microsatellite studies, are not a factor in this dataset. In comparisons between the West Fork Canyon and Indian Creek Canyon samples of the Animas Mountains, homogeneity of allele frequencies, minimal genetic distance, and exceptionally low F_{ST} all suggest little or no population genetic structure. Estimates of migration rate appear to differ dramatically, but from a functional perspective, $Nm = 3$ is more than sufficient to effect homogeneity of allele frequencies. Migration rates within the Sierra San Luis are probably comparable to the Animas Mountains, given the continuity and high quality of habitat in the former. Migration rates within the Peloncillo population may not be comparable to

TABLE 5. Distribution of alleles unique to each population of *Crotalus willardi obscurus*. T = total number of alleles from a population, U is the number of alleles unique to the population, and the number of these that occur at a frequency ≥ 5% is indicated in parentheses, and % = (U/T) x 100.

	San Luis			Peloncillo			Animas		
Locus	*T*	*U*	%	*T*	*U*	%	*T*	*U*	%
*Cw*A14	8	4 (4)	50	3	1 (1)	33	7	2 (1)	29
*Cw*A29	11	6 (3)	55	5	1 (1)	20	5	1 (0)	20
*Cw*B6	7	2 (1)	29	4	1 (0)	25	5	1 (0)	20
*Cw*B23	13	5 (1)	39	7	3 (2)	43	12	4 (2)	33
*Cw*C24	18	5 (3)	28	15	1 (0)	7	24	6 (0)	25
*Cw*D15	7	2 (2)	29	5	0	0	5	1 (1)	20
Scμ01	9	3 (1)	33	4	0	0	11	5 (4)	46
Scμ07	1	—	—	1	—	—	2	1 (1)	50
Scμ11	12	5 (2)	42	6	0	0	10	3 (2)	30
Total	**86**	**32 (17)**	**37**	**50**	**7 (4)**	**14**	**81**	**24 (10)**	**30**

TABLE 6. Measures of population differentiation among samples and populations of *Crotalus willardi obscurus*. F_{ST} (Weir and Cockerham, 1984) is above the diagonal and genetic distance (Nei, 1978) is below the diagonal. ICC = Indian Creek Canyon and WFC = West Fork Canyon in the Animas Mountains.

	Animas (WFC)	Animas (ICC)	Peloncillo	San Luis
Animas (WFC)	—	0.004	0.211	0.139
Animas (ICC)	0.035	—	0.203	0.129
Peloncillo	0.788	0.788	—	0.144
San Luis	0.558	0.544	0.446	—

	Animas	Peloncillo	San Luis
Animas	—	0.203	0.133
Peloncillo	0.780	—	0.144
San Luis	0.543	0.446	—

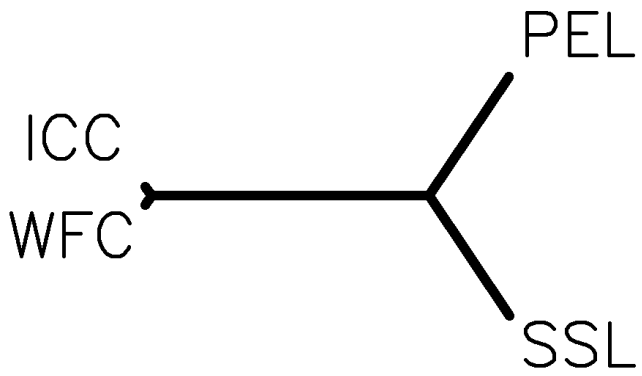

FIG. 3. UPGMA phylogenetic tree of relationships among *Crotalus willardi obscurus* populations based on genetic distance (Nei, 1978). ICC and WFC represent the Indian Creek Canyon and West Fork Canyon samples in the Animas Mountains, while PEL and SSL represent the Peloncillo Mountains and Sierra San Luis samples.

values calculated for the Animas population, because population densities are probably much lower (see below), habitat is of lower quality, and habitat patches are much more fragmented and widely spaced.

Comparison of number of alleles detected, average expected heterozygosity, and private alleles among populations all suggest decreased variability in the Peloncillo population relative to the other two populations. Additionally, the Peloncillo population tested positive in two statistical assays for population bottlenecks. Both methods are based on the TPM, although the method of Cornuet and Luikart (1996) relies on differential rate of decline in two measures (see above) using a mutation model-dependent (TPM in this case) estimation, whereas the method of Garza and Williamson (2001) compares two measures directly and contrasts the ratio with a critical value (M_c) from a user-specified parameterization of the TPM. Both methods require stout sample sizes in order to have much power, and the Peloncillo sample size is marginal for either test. A minimal sample of 15 individuals and 10 loci is recommended for the Wilcoxon test in BOTTLENECK and a sample of 25 or twice the number of alleles at the most polymorphic locus is recommended for M. We calculated M_c using two different sets of mutation parameters. In the first case, we set the proportion of stepwise mutations (p_s) to 0.90 and the average increase in non-stepwise mutations (Δ_g) to 3.5 (parameters recommended by the authors). For a more conservative comparison, we set p_s = 0.80 and left Δ_g =3.5. In all cases θ = 10 and sample size and number of loci were fixed. Under these parameterizations, M_c = 0.616, 0.681, and 0.648 (recommended) and 0.542, 0.623, and 0.580 (conservative) for the Peloncillo, Animas, and San Luis populations, respectively. Using the parameterization recommended by the authors, the Peloncillo and Animas populations both tested positive for recent bottlenecks. None of the populations tested positive under the more conservative parameterization. Garza and

Williamson (2001) presented measures of the statistic *M* for 12 natural populations with stable histories and eight populations with documented histories of severe population reductions or founder events. Stable populations ranged from 0.823–0.926 whereas founder, island or reduced populations ranged from 0.599–0.693. The values of *M* for all three *C. w. obscurus* populations fall in the latter range, with the exception of the Peloncillo population, which falls below it. As a whole these tests suggest that reductions in population size have, or are, reducing variability in the Peloncillo population, although given the uncertain mode of microsatellite mutation and the limited sample size, it is not possible to characterize the nature and extent of this decline.

Interestingly, under all but the most conservative parameterizations of TPM, the Animas population also tested positive for bottlenecks using *M* and approached significance ($P = 0.08$) in the Wilcoxon test. The Animas Mountains are higher in elevation than the Peloncillo Mountains, and have higher quality habitat but are quite limited in area (< 30 km^2 of habitat). Thus, this population might be more vulnerable to stochastic environmental catastrophes such as fire or prolonged and severe drought.

Despite decreased variability relative to its neighbors and possible bottlenecks, the Peloncillo population is not genetically impoverished at these neutral markers. Some of these measures (e.g. number of alleles) are sensitive to low sample size (especially with highly variable loci). In addition, overall F_{IS}, a measure of inbreeding (in the absence of null alleles and Wahlund effects), is not unreasonably high and is comparable with overall F_{IS} in the Animas Mountains. Indeed, samples in the Peloncillo Mountains were collected over several canyons (as compared to one canyon in the Sierra San Luis and two canyons in the Animas Mountains), raising the possibility that a Wahlund effect is contributing to F_{IS} in the Peloncillo and Animas populations. Regardless of genetic diversity, low capture rates (294 person-hours/snake) suggest

a very low-density population in the Peloncillos, comprised of few snakes dispersed among fragmented habitat. Capture rates in the Animas Mountains (37 person-hours/snake) and Sierra San Luis (11 person-hours/snake) were much higher than those recorded in the Peloncillo Mountains. Although strong evidence for inbreeding in the Peloncillo is lacking at these neutral markers, we found that two (of 18) individuals (11%) captured in recent years exhibited abnormalities of the rattle that appear to be congenital. We have not observed rattle abnormalities in the Animas Mountains (N = 160) or Sierra San Luis (N = 29).

Even at moderate densities, selection in rattlesnake mating systems appears to act significantly on male mate-searching abilities (Duvall et al., 1992). In low density fragmented populations, where the ability to find mates is compromised, an Allee Effect (Allee, 1958) does not seem improbable. Anecdotal evidence suggests that mate-finding in the Peloncillo population is problematic. First, the population was discovered from a *C. willardi* X *lepidus* hybrid (Campbell et al. 1989), the only documented natural hybrid between these two broadly sympatric and syntopic species. Rare hybridization events between syntopic taxa often result from breakdown of pre-mating isolating mechanisms; a consequence of low population density in one of the two taxa. Paternity analysis of a litter of eight born in the Peloncillo offers a second line of evidence that the pool of potential mates in the Peloncillo population is small. Using genotypes from this litter and all male snakes captured in Clanton Draw, we calculated a 98% probability of paternity for a male snake captured the previous year in the same canyon. Over the course of four years, only two male snakes had been captured in this drainage, and one of these sired the litter. Inter-canyon migration in the Peloncillo Mountains may be essential for population viability in a fragmented low-density system.

Population Differentiation

All measures of population differentiation illustrate that a high proportion of genetic variance in *C. w. obscurus* is partitioned among geographically discrete island populations. All loci significantly differed in allele frequencies among populations and all had high levels of unique and private alleles, suggesting that these populations have been isolated long enough to allow genetic drift to effect significant divergence. While a high proportion of unique alleles can suggest significant divergence between populations, it can also result from missing low frequency alleles due to inadequate sampling of alleles (2*N*) relative to the number of alleles detected overall. In this study, allelic diversity was high, but generally was less than a quarter the number of alleles sampled, with the exception of locus *Cw*C24 (Table 3). Nevertheless, in order to account for this effect we contrast the number of private alleles found in this study with previous studies. Private alleles occurred in proportions equivalent to those reported for Eastern Massasauga (Gibbs et al., 1997). In the aforementioned analysis of microsatellite variation among 6 populations of Bighorn Sheep, Gutiérrez-Espeleta et al. (2000) did not detect any private alleles.

Overall F_{ST} for these populations (0.16) is relatively high when considered in the context of the results of other microsatellite studies at wider spatial scales. For example, Gibbs et al. (1997) obtained an overall F_{ST} of 0.164 in a comparisons among five populations of Massasauga rattlesnakes from the Great Lakes region, with the furthest populations over 600 km apart. Likewise, Massasauga from the desert southwest, separated by 410 km had an F_{ST} of 0.127. Gutiérrez-Espeleta et al. (2000) found that $F_{ST} = 0.204$. The samples referenced herein are separated by < 50 km in all cases, and the Sierra San Luis is separated from both the Peloncillo and Animas mountains by as little as 5–10 km of unsuitable habitat. Genetic distances calculated

here are likewise high, when considered in the context of the literature. For example, Gibbs et al. (1997) calculated a distance of 0.799 between the Cicero, New York and Springfield, Ohio populations of Massasauga, while we calculate a comparable distance of 0.780 between the Animas and Peloncillo populations. A linear distance of 10 to 20 km separates the Animas and Peloncillo populations, whereas the two Massasauga populations are separated by over 600 km.

Relationships among populations as hypothesized in the unrooted UPGMA tree based on genetic distance should be interpreted carefully. Although the Animas samples are clearly most closely related to one another, the relationships among the three montane populations are less certain. Genetic distance increases linearly with time since divergence of two populations. However, genetic distance is sensitive to population size, and can increase rapidly if one or both of the populations experiences a significant decrease in population size (Hedrick, 1999). In the present example, the Peloncillo population exhibits reduced diversity and the statistic *M* suggests a recent bottleneck, creating a context for inflated genetic distance measures in pair-wise comparisons. Nevertheless, pair-wise genetic distances between the populations suggest that the Sierra San Luis and Peloncillo populations are more closely related than the Animas and San Luis populations and that the Animas and Peloncillo populations are most distantly related. Even if low sample size in the Peloncillo population is inflating distance measures in pair-wise comparisons, it appears that the trend among populations is valid. These patterns fit intuitive expectations given the biogeography of the region. The Peloncillo Mountains and Sierra San Luis are separated only by a series of low (ca. 1670 m) dissected hills currently dominated by a mosaic of grassland and oak woodland that is marginal habitat for *C. w. obscurus*. Given paleoecological evidence from the region, it seems reasonable that within the last 10,000 years these hills supported more extensive woodlands and possibly a contiguous San Luis-Peloncillo

population of *C. w. obscurus*. A low elevation (1677 m) pass dominated by desert scrub and xeric grassland may provide a more extensive barrier between the Sierra San Luis and Animas Mountains. Accordingly, the vicariant event separating the latter populations may have occurred earlier, perhaps near the end of the last pluvial period, if not before. In addition to their association with woodlands, *C. w. obscurus* is saxicolous and seldom found far from rocks (Armstrong and Murphy, 1979; McCranie and Wilson, 1987; Holycross et al., in press). Consequently these rocky intermontane passes may have provided reasonable habitat for the species prior to xerification and changes in the biotic community. In contrast, the broad flat grasslands of the Animas Valley separate the Animas and Peloncillo mountains. This barrier is substantially lower in elevation (1500 m) and more sustained than the aforementioned mountain passes. Even if woodlands covered the Animas Valley during recent glacial episodes, it is uncertain whether or not the basin would have afforded suitable habitat due to the lack of other habitat components such as rocks and slopes.

Most intraspecific phylogenies and phylogeographic scenarios (McCranie and Wilson, 1987; Barker, 1992) place *C. w. obscurus* as sister to *C. w. silus* and/or suggest that *C. w. obscurus* is a recently derived subspecies in the clade. *Crotalus w. obscurus* is separated from *C. w. silus* (in the Sierra el Tigre and beyond) by a low elevation valley cut by the Rio Bavispe. The two subspecies differ dramatically in background coloration and facial patterns, suggesting significant divergence. When ancestral *C. willardi* crossed the Rio Bavispe and occupied the Sierra San Luis, barriers to further dispersal into the Animas and Peloncillo Mountains should have been minimal to nonexistent. In the context of 1) the biogeographic history of the region, 2) the low vagility and habitat specificity of the organism and 3) these population genetic data, vicariance seems a more probable explanation of the genetic diversity observed among

populations than colonization of the Animas and Peloncillo populations across barriers. Although allele frequencies differ significantly, the distribution of allele sizes overlaps broadly among populations, and diminished allelic diversity in the Peloncillo population generally occurs throughout the range of allele sizes or is a subset of variation found in the San Luis population. In the context of the generation lengths (ca. 3 years) and mutation rates (ca. 5×10^{-4}/locus/generation) that apply here, if *C. willardi* crossed barriers to occupy the two northern ranges, then colonized populations should exhibit reduced variability due to founder effects and/or show evidence of divergent allele distributions at some loci. Subsequent to invasion of this V-shaped complex of mountain ranges, tips of the "V" may have begun to diverge *in situ*, with subsequent vicariant events creating the context for independent evolution of all three populations. Estimation of migration rate among populations is low, with $Nm = 0.66$, or less than one migrant/generation. The private alleles method predicts a decrease in log*Nm* as a linear function of the average frequency of private alleles. This method does not demonstrate gene flow is occurring, but rather provides an index of migration rates assuming that gene flow is occurring. Low *Nm*, such as that estimated here, is not inconsistent with the absence of extant gene flow.

CONSERVATION IMPLICATIONS

These data, biogeographic history of the region, and the low vagility and habitat specificity of this species all suggest that the populations occupying these three mountain ranges are genetically isolated and currently on independent evolutionary trajectories. Variation among populations at these neutral loci is largely a consequence of genetic drift. Although this study does not demonstrate adaptive variation among populations, it predicts substantial potential for local adaptation, provided that selection pressures vary significantly among populations

(Hedrick, 1999; Hedrick et al., 2001). Disparate ecological conditions in the Peloncillo Mountains relative to the Sierra San Luis and Animas Mountains suggest that this is not an unreasonable supposition. We recommend that each mountain population be managed as an evolutionary significant unit (ESU; Waples 1995) or distinct population segment (DPS). Data from the Animas Mountains suggests genetic cohesiveness and high levels of gene flow within mountain ranges, although this generality may not apply to the Peloncillo Mountains.

Comparatively low levels of diversity in several genetic parameters and statistical evidence for a bottleneck suggest reductions in the size of the Peloncillo population. In addition to low genetic variability, field studies also suggest this population is exceptionally small. While transplantation of snakes from neighboring populations does not appear to be necessary or advisable from a genetic perspective, supplementation might be advisable from a demographic perspective (see Lande, 1988). Ideally, supplementation could be effected via a captive breeding program specific to the Peloncillo population (source animals from the Peloncillo Mountains). However, captive breeding programs are fraught with uncertainty, and the Peloncillo population may not be able to sustain repeated or substantial harvest, even for captive breeding. A pilot program using < 20 founder animals might be designed to assess the feasibility of captive breeding and survival rates of naïve snakes upon repatriation. In the event that using snakes from the Peloncillo Mountains is not possible, these data and the biogeography of this mountain complex suggest that the San Luis population is the most suitable alternative source population for demographic supplementation.

More than a century of fire suppression has artificially inflated fuel loads in *C. w. obscurus* habitat. Poorly managed prescribed fire and naturally ignited fire are currently a direct threat to the habitat of this species, and have already resulted in significant loss of demonstrably

occupied habitat (Smith et al., 2001). Exceptionally low population densities and fragmented habitat suggest that maintaining habitat patches is essential to population viability. Preservation of limited woodland habitat and a cautious and conservative approach to the reintroduction of fire should receive priority ranking in management decisions and funding allocations. With continued attrition of woodland habitat, captive breeding for demographic supplementation is moot, however, captive propagation would still serve the function of retaining a population in refugium.

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APPENDIX

ALLELE FREQUENCIES IN SAMPLES OF *Crotalus willardi obscurus* BY LOCUS.

ICC and WFC indicate Indian Creek Canyon and West Fork Canyon demes, respectively.

	San Luis	Peloncillo	Animas (pooled)	Animas (ICC)	Animas (WFC)
***Cw*A14**					
147	0.034		0.046	0.083	0.017
149	0.190				
153	0.052				
155	0.069				
159	0.086		0.157	0.167	0.150
161			0.278	0.167	0.367
163	0.155				
165	0.259	0.667	0.306	0.375	0.250
167	0.155	0.194	0.009	0.021	
169		0.139	0.185	0.188	0.183
175			0.019		0.033

***Cw*A29**					
160	0.034		0.111	0.146	0.083
170	0.466		0.796	0.750	0.833
172	0.138	0.528			
174		0.167	0.074	0.063	0.083
176			0.009	0.021	
178	0.017				
182	0.017				
184	0.121				
186	0.034	0.167			
188		0.083			
190	0.034	0.056	0.009	0.021	
192	0.034				
194	0.052				
196	0.052				

***Cw*B6**					
98	0.241	0.056			
118	0.052				
120	0.017				
122	0.310	0.444	0.287	0.354	0.233
124	0.052		0.250	0.292	0.217
126	0.310	0.472	0.361	0.313	0.400
128	0.017		0.065	0.042	0.083
130			0.037		0.067
134		0.028			

***Cw*B23**					
225			0.094	0.104	0.086
227			0.226	0.250	0.207
231			0.009	0.021	
233		0.111			
241			0.009	0.021	
245	0.034		0.066	0.083	0.052
247	0.017				
249	0.207		0.245	0.188	0.293
251	0.155	0.056	0.113	0.167	0.069
253	0.017		0.075	0.063	0.086
255	0.017	0.361	0.066	0.021	0.103
257	0.138				
259		0.111			
261	0.017				
263		0.028			
265	0.017		0.038	0.063	0.017
267	0.034				
269	0.121	0.056	0.019		0.034
271	0.207	0.278	0.038	0.021	0.052
275	0.017				

***Cw*C24**					
229	0.017	0.056			
232	0.259	0.056			
235	0.052		0.019	0.022	0.017
238			0.019	0.022	0.017
241			0.019	0.022	0.017
244			0.019	0.022	0.017
247		0.028	0.009	0.022	
250	0.017	0.028	0.047	0.109	
253	0.052	0.111	0.142	0.152	0.133
256	0.052		0.047		0.083
259	0.052		0.047	0.022	0.067
262		0.028	0.019	0.043	
265	0.017	0.139	0.189	0.130	0.233
268	0.017	0.139	0.085	0.065	0.100
271		0.056	0.028	0.043	0.017
274	0.086	0.139	0.047	0.022	0.067
277		0.056	0.028	0.022	0.033
280	0.017	0.083	0.019	0.022	0.017
283			0.009	0.022	
286	0.052	0.028	0.019	0.022	0.017

289			0.009	0.022	
292		0.028			
295	0.069		0.047	0.065	0.033
298	0.034		0.019	0.022	0.017
301	0.086				
304		0.028	0.009	0.022	
310	0.017		0.094	0.087	0.100
313			0.009		0.017
316	0.017				
319	0.086				

***Cw*D15**					
132	0.241				
138	0.138	0.111	0.519	0.583	0.466
141	0.293				
144	0.034	0.222	0.236	0.188	0.276
147	0.121	0.139	0.151	0.125	0.172
153	0.103	0.444	0.047	0.021	0.069
156	0.069	0.083			
159			0.047	0.083	0.017

***Scu*01**					
158	0.143				
166	0.018		0.066	0.104	0.034
177	0.357		0.047	0.063	0.034
178	0.268	0.083			
181	0.018		0.198	0.167	0.224
182	0.054	0.639			
183			0.009	0.021	
186	0.089		0.085	0.042	0.121
191	0.036				
194			0.075	0.146	0.017
196			0.057	0.083	0.034
198			0.047		0.086
200			0.283	0.271	0.293
202		0.167	0.009	0.021	
204		0.111	0.123	0.083	0.155
208	0.018				

***Scu*07**					
146	1.000	1.000	0.500	0.478	0.517
148			0.500	0.522	0.483
***Scu*11**					
170	0.315				
172			0.028	0.042	0.017
175	0.056				
176			0.283	0.229	0.328
181	0.037				
182	0.019		0.075	0.063	0.086
184	0.111		0.057	0.104	0.017
194	0.185	0.222			
196	0.037				
198	0.056	0.083			
202	0.074	0.028	0.132	0.146	0.121
204	0.056	0.056	0.113	0.104	0.121
206		0.444	0.066	0.063	0.069
210		0.167	0.104	0.063	0.138
212			0.104	0.104	0.103
214	0.019		0.038	0.083	
216	0.037				